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Entry and replication of Japanese encephalitis virus in cultured neurogenic cells

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Summary

The entry mode and growth pattern of Japanese encephalitis (JE) virus in mouse neuroblastoma N18TG2 cells and mouse neuroblastoma x rat glioma NG108-15 hybrid cells were studied by electron microscopy. At two minutes after inoculation. JE virions adsorbed onto and directly penetrated through the plasma membrane of the hybrid cells, whereas virions did not adsorb nor entered the neuroblastoma cells. Correspondingly, the hybrid cells showed assembling progeny JE virions in the cisternae of rough endoplasmic reticulum (RER) 1 day postinoculation (p.i.) although virions were rarely found on the following days during the experiment. On the other hand, progeny virions did not assemble in the RER cisternae of the neuroblastoma cells throughout the experiment. The morphologic observations, therefore, suggest that (a) the hybrid cells express JE-virus receptors which facilitate the viral attachment onto and entry into the cells, while the neuroblastoma cells do not and (b) JE virus replicates very poorly after the entry into the hybrid cells while it does not replicate at all in the neuroblastoma cells. The virus titrations of the media of the neuroblastoma and hybrid cell cultures showed only titers indicative of residual virus of the inoculum that progressively decreased during the experiment. The present results show therefore that of the two neurogenic cell culture lines studied only the hybrid cell line can be used for the study of viral entry and replication, although it is not suited for virus production. Possible reasons for the poor replication of JE virus in the hybrid cells are discussed.

Japanese encephalitis virus; Mouse neuroblastoma N18TG2 cell; Mouse neuroblastoma x rat glioma NG108-15 hybrid cell; Viral entry; Viral replication; Electron microscopy

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Introduction

Japanese encephalitis (JE) virus is a member of the Flaviviridae family that is transmitted to vertebrate hosts by vector mosquitoes (Buescher and Scherer, 1959; Buescher et al., 1959; Scherer et al., 1959). In laboratory mice, following intracerebral inoculation the virus infects neurons of the central nervous system selectively (CNS), and replicates and matures exclusively in the neuronal secretory system including the rough endoplasmic reticulum (RER) and the Golgi apparatus (Hase et al., 1987, 1990). In light of the selective neurotropism, it is assumed that neurons in the CNS express surface receptors that have a strong affinity to JE virus; yet, nothing is known about the neuronal receptor for JE virus. Since viral entry modes are usually studied most effectively in vitro cell-culture systems, progress in the study of JE virus-neuron interaction for entry is especially hindered by the lack of an adequate in-vitro host-cell model that mimics the in-vivo infection. Although cell-culture lines of neuronal origins are available, there are no studies that report successful infections of neurogenic cell lines with JE virus. In this study, we investigated the entry mode and replication pattern of JE virus in mouse neuroblastoma and mouse neuroblastoma x rat glioma hybrid cell lines.

Materials and Methods

Virus

JE virus, Chinese strain SA_{14}, was used in this study. The seed virus was passed two times intracerebrally in suckling mice and once in *Aedes albopictus* C6/36 cells. The virus harvest fluids from the last passage were pooled and clarified by centrifugation at 600 × *g* for 10 min, and the virus particles were pelleted at 63 000 × *g* for 2 h. The pellet was resuspended to 1/100 original volume in 0.01 M Tris-HCl, pH 7.8, containing 0.15 M NaCl and 0.05 M EDTA. Three ml of the suspension were layered on a 27 ml gradient of 5–25% (w/v) sucrose and centrifuged for 2.5 h at 65 000 × *g* in a swinging bucket rotor. One ml fractions were collected and screened for hemagglutinating activity (HA). The fractions with the highest HA activity were pooled, aliquoted, and stored at −70°C until use.

Cell culture

Mouse neuroblastoma N18TG2 cells and mouse neuroblastoma x rat glioma NG108-15 hybrid cells were generous gifts of Dr. Marshall Nirenberg (NIH, Bethesda, MD). The hybrid NG108-15 cells were grown in 25 cm^2 tissue culture flasks at 37°C in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) (Grand Island Biological Co., Grand Island, NY), supplemented with 5% fetal calf serum, 0.1 mM hypoxanthine, 16 μM thymidine, and 1 μM

aminopterine with 100 U of penicillin and 100 μg of streptomycin per ml in a humidified atmosphere containing 10% CO_2. The neuroblastoma N18TG2 cells were grown as above supplemented with 10% fetal calf serum and the antibiotics without hypoxanthine, thymidine, and aminopterine. All experiments were performed with 80–90% confluent cultures on day 5 following subculture.

Viral inoculation

The neuroblastoma and hybrid cell cultures were inoculated with JE virus at an MOI of 50 and incubated at 37°C in a humidified atmosphere containing 10% CO_2 for 2 min for the viral entry study and for 1, 2, and 3 days for the viral replication study. At the end of each period, the culture medium was removed, and the cells were immediately suspended in 1/2 strength Karnovsky fixative and fixed overnight at 4°C. For the virus titration, 0.2 ml of the culture medium was withdrawn at various intervals up to 3 days postinoculation (p.i.), and infectious virus titers determined by a standard plaque assay (Eckels et al., 1976).

Electron microscopy

After fixation in Karnovsky fixative, the cell samples were washed in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% cacodylate-buffered osmium tetroxide, dehydrated, and embedded in epon 812. Thin sections were cut on a LKB Ultrotome, Nova, placed on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss SM109 or Philips CM12 electron microscope.

Results

For the study of viral entry at 2 min p.i., the surfaces of 50 cells in each sample were scanned by transmission electron microscopy at the magnification of × 20000 for adsorbed virions. A majority of the hybrid cells demonstrated adsorbed virions on their surfaces with the total count being 165 virions or the average of 3.3 virions per cell. In contrast, none of the neuroblastoma cells examined showed adsorbed virions on their surfaces. In the hybrid cells, JE virions were seen, adsorbed on microvilli, cytoplasmic processes, and on the cell surface (Figs. 1,2). JE virions measured 40 to 45 nm in diameter and showed the central, electron-dense nucleocapsid covered by the membrane envelope having fine, short spikes. Adsorbed virions appeared to penetrate through the host plasma membrane into the cytoplasm directly, as described in the entry of JE virions into C6/36 mosquito cells (Hase et al., 1989a,b). As in the case of JE virion entry into C6/36 mosquito cells, we could divide the entry process into three stages: stage one at which virions attached to the plasma membrane by their envelope spikes; stage 2 at which the viral envelope and the plasma membrane approximated each other, causing dissolution of the plasma membrane at the sites; and stage 3 at which virions entered the cytoplasm through the membrane

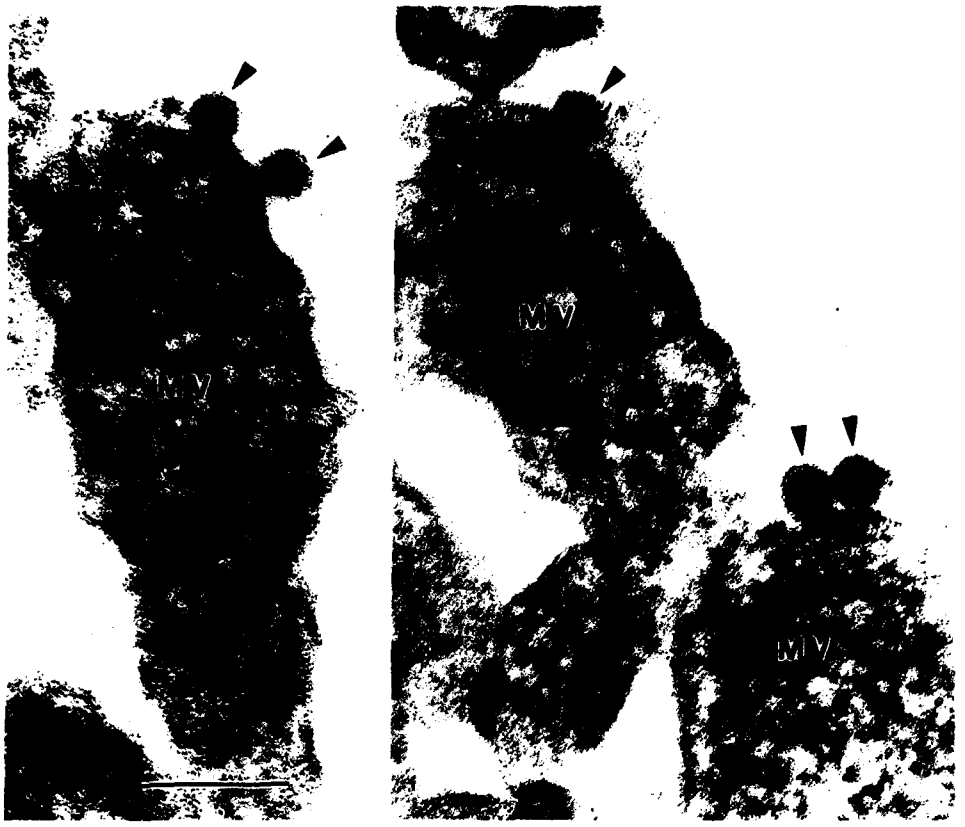

Fig. 1. JE virions (arrowheads) adsorbed on microvilli (MV) of a hybrid cell, 2 min p.i. Bar=100 nm.

disruptions and disintegrated themselves (uncoated). No coated pits developed at the virion-adsorption sites, and no evidence that virions entered the hybrid cells by receptor-mediated endocytosis was found.

For the study of viral replication on 1, 2, and 3 days p.i., assembling progeny virions were sought specifically in the cisternae of the RER, where JE virions are known to assemble (Hase et al., 1987,1990). Both the neuroblastoma and the hybrid cells were found to have irregular shape with large nuclei in the center. The cytoplasm contained mostly granular material consisting of free ribosomes and polysomes and other proteinaceous material. Varying numbers of mitochondria and cytoplasmic vesicles and vacuoles were also observed. The RER segments were scattered sparsely and irregularly in the cytoplasm. The Golgi apparatus was not prominent and usually difficult to find. Cells in mitosis were found occasionally in both cell cultures. Assembling virions were not found in the RER cisternae of the neuroblastoma cells during the 3-day period of experiment. In contrast, assembling progeny JE virions were found sporadically in the cisternae of RER of the hybrid cells on day one p.i. (Fig. 3A,B). Generally, the RER

Fig. 2. JE virions (arrowheads) at various stages of direct penetration through the plasma membrane of a hybrid cell, 2 min p.i. M, mitochondrion; N, nucleus; R, rough endoplasmic reticulum. Bar=100 nm.

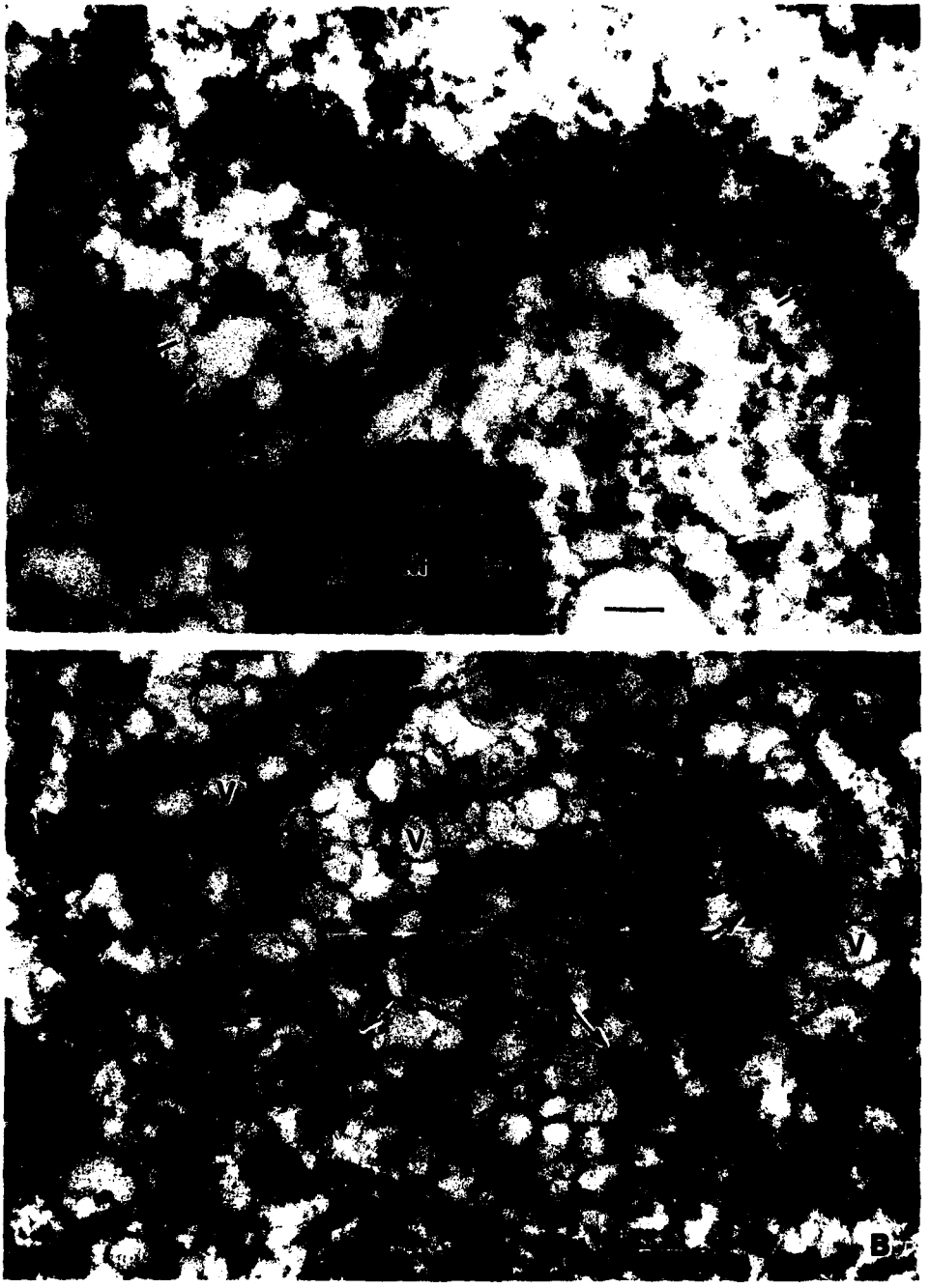

Fig. 3. Assembling JE virions in the RER cisternae of hybrid cells, 1 day p.i. A. Assembling virions (arrows) in the cisternae of lamellar RER segments. Bar=100 nm. B. Assembling virions (arrows) and ER vesicles (V) in the moderately dilated RER cisternae. Bar=100 nm.

cisternae did not show any remarkable dilation such as seen in those of JE virus-infected mouse brain neurons (Hase et al., 1987,1990). Accordingly, assembling virions were frequently sandwiched between the two lamellar RER membranes (Fig. 3A). The ER vesicles were occasionally found in some mildly to moderately dilated RER cisternae (Fig. 3B). The ER vesicles are characteristic membrane vesicles of varying sizes that occur in the RER cisternae in connection with flavivirus replication (Hase et al., 1989c). Assembling virions were found less frequently in the RER cisternae of the hybrid cells on day two p.i. and not at all on day three p.i. We failed to find any virions within transport vesicles or in the Golgi saccules.

The JE virus titers in the media of the neuroblastoma and hybrid cell cultures during the experiment are shown in Fig. 4. The virus titers in both cultures declined progressively indicating absence of virus production in the media.

Discussion

It is generally considered that the attachment of viruses to specific receptors on the plasma membrane of host cells is the first step necessary for productive viral infection; this, also, is believed to constitute one of the major determinants of viral tropism and pathogenesis (Holland, 1961; Longberg-Holm and Phillipson, 1978; Tardieu et al. 1982). In the infection of mammalian hosts, JE virus shows tropism to neurons in the CNS, suggesting that neurons in the CNS express on

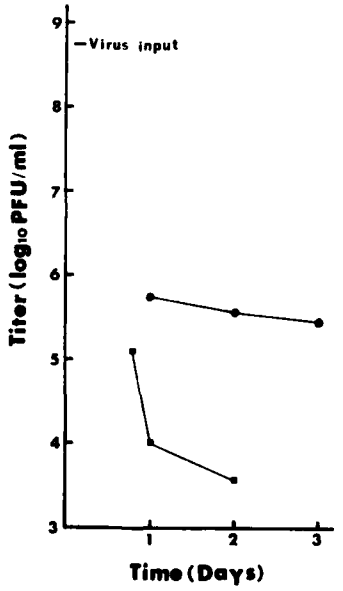

Fig. 4. JE virus titers in the media of the neuroblastoma-cell and hybrid-cell cultures. Neuroblastoma-cell cultures, •——•; Hybrid-cell cultures, ■——■.

their surfaces specific receptors that have strong affinity to JE virus (Oyanagi et al., 1969; Hase et al., 1990). The present study demonstrated that at 2 min p.i. many JE virions adsorbed on the hybrid cells while JE virions did not adsorb on the neuroblastoma cells. Moreover, the virions adsorbed onto the hybrid cells showed varying stages of penetration through the host plasma membrane into the cytoplasm. The above findings suggest that the hybrid cells express specific surface receptors that bind to JE virus for viral entry while the neuroblastoma cells do not express these surface receptors. It has been shown that the hybrid cells resemble neurons in the expression of various surface receptors such as morphine and acetylcholine receptors (Klee and Nirenberg, 1974; Hamprecht, 1977; Nathanson et al., 1978; Ray et al., 1989). In this respect, the hybrid cells apparently express the receptor for JE virus in contrast to the neuroblastoma cells that do not. At present, little is known about neuronal receptors for various neurotropic viruses. Acetylcholine receptors are suspected to function as receptors for rabies virus (Lentz et al., 1982; Burrage et al., 1985). In the search for the neuronal receptors for JE virus, the hybrid cells and the neuroblastoma cells may be used as receptor-positive and receptor-negative cells respectively.

The growth pattern of JE virus in mouse brain neurons has recently been clarified. JE virus undergoes trans-type maturation in that viral RNA replication and protein synthesis occur on smooth and rough endoplasmic reticulum respectively, with resultant release of the products into the cisternae (Hase et al., 1987,1989c). The assembly of progeny virions occurs exclusively in the RER cisternae, and the assembled virions are released extracellularly through the cellular secretory channel via the Golgi apparatus. In this type of viral maturation, therefore, localization of assembling progeny virions in the RER cisternae becomes a hallmark of intracellular viral replication. The present study demonstrated that, after entry by direct penetration, JE virus replicated in the RER cisternae of the hybrid cells, indicating that JE virus underwent trans-type maturation in the hybrid cells. On the other hand, no virions entered the neuroblastoma cells shortly after the inoculation, and no virions were found in the RER cisternae 1, 2, and 3 days p.i., thus presenting evidence that viral replication did not occur in these cells.

Although both viral entry and viral replication were observed in the hybrid cells, progeny virions appeared only sporadically within the cisternae of irregularly scattered segments of RER. In the infection of mouse brain neurons with JE virus, on the other hand, it has been reported that large numbers of progeny virions appear within dilated cisternae of hypertrophic RER of infected neurons (Hase et al., 1987, 1990). In this regard, JE virus replication in the hybrid cells seems to be far more restricted than that in mouse brain neurons. The comparison of JE virus replication in the hybrid cells in vitro and in mouse brain neurons in vivo, therefore, may suggest that there are intracellular factors that influence viral replication after viral entry. Wunner et al. (1984), in search of a possible explanation for the strict neurotropism that rabies virus exhibits in vivo, have reported that attenuated rabies virus recognizes and competitively binds to the same receptor as does wild-type virulent virus. This suggests that the receptor site utilized in culture does not differentiate between rabies viruses of patho-

genic and nonpathogenic phenotypes and that the pathogenic discrimination in vivo may come after initial viral attachment. During viral growth there are multiple points where a viral replication cycle can be aborted. The reason for poor JE virus replication after its entry into the hybrid cells is not clear at present. Nonetheless, in light of the dependence of trans-type flavivirus maturation on the host-cell secretory system, it is interesting to speculate that JE virus, a trans-type virus, replicates optimally in brain neurons that are stable and non-dividing and are engaged in active secretion. The hybrid cells in this study were actively dividing cells whose secretory activity was in question. The secretory system of these cultured cells most likely went through breakdown and regeneration in accordance with the mitotic cycle of the cells. Morphologically, brain neurons are equipped with well developed RER and Golgi apparatus; on the other hand, the hybrid cells in culture usually showed sparse, irregularly scattered segments of RER and poorly developed Golgi apparatus. It is possible, therefore, that the functional state of host cells may play an important role for the replication of JE virus after the entry.

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